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FORM PTO-1390 (REV 5-93)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 146.1275
<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO (If known, see 37 C.F.R. 1.51) <b>08/945459</b>
INTERNATIONAL APPLICATION NO PCT/JP96/01062		INTERNATIONAL FILING DATE April 19, 1996
PRIORITY DATE CLAIMED 4/19/95 & 11/17/95		
TITLE OF INVENTION A NOVEL PROTEIN AND PROCESS FOR PREPARING THE SAME		
APPLICANT(S) FOR DO/EO/US FUSAO MAKISHIMA et al		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. to 16. below concern other document(s) or information included:		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information		
Also Enclosed: Seven (7) sheets of drawings Form PCT/IB/306 (March 1994)		

17 ☒ The following fees are submitted.

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO .. . \$830.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)

\$640.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482)

but international search fee paid to USPTO (37 CFR 1.445(a)(2)).. \$710.00

Neither international preliminary examination fee (37 CFR 1.482) nor

international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$950.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)

and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$90.00

ENTER APPROPRIATE BASIC FEE AMOUNT = \$1070.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

Claims	Number Filed	Number Extra	Rate
Total Claims	15 -20 -	0	X \$22.00
Independent Claims	1 -3 -	0	X \$74.00
Multiple dependent claims(s) (if applicable)	0	+	\$230.00

TOTAL OF ABOVE CALCULATIONS = \$1070.00

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).

SUBTOTAL = \$1070.00

Processing fee of \$130.00 for furnishing the English translation later the ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

TOTAL NATIONAL FEE = \$1070.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

TOTAL FEES ENCLOSED = \$1070.00

Amount to be:  
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- a. ☒ A check in the amount of \$ 1070.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2275. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:  
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19,683

REGISTRATION NUMBER

12/09/97  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:  
F. MAKISHIMA et al  
Serial No.: 945,459  
Filed: October 20, 1997  
For: NOVEL PROTEIN...THE SAME

600 Third Avenue  
New York N.Y. 10016  
December 10, 1997

COMPLETION OF APPLICATION

Asst. Commissioner for Patents  
Washington, D.C. 20231

Sir:

Applicants are submitting herewith the executed declaration to replace the unexecuted declaration filed with the application. Also submitted is a check for \$130.0 for the completion fee. The filing of the application is now complete and it is requested that the application be forwarded to the group for examination in due course.

Respectfully submitted,  
Bierman, Muserlian and Lucas

By: Charles A. Muserlian  
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CAM:ds  
Enclosures

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08/945459/25/P006DS

DATE OF FILING

Title of the InventionA novel protein and process for preparing the sameBackground of the Invention

## 1. Field of the Invention

This invention relates to a protein having amino acid sequence in SEQ ID No.:1 of the Sequence Listing derived from MP52. The invention also relates to a homodimer protein of said protein and a pharmaceutical composition for treating cartilage and bone diseases containing the dimer protein as an active ingredient. The invention also relates to a process for preparing the above described protein in a large amount and with a high purity by culturing *E. coli* which was transformed with a plasmid containing a DNA sequence capable of expressing the above described protein. The invention further relates to a method for treating cartilage and bone diseases, which comprises administering to a human a pharmaceutical composition containing, as an active ingredient, an effective amount of the homodimer protein.

## 2. Description of the Prior Art

Pharmaceutical compositions comprising vitamin D<sub>3</sub>, calcitonin, estrogen or their derivatives as well as bisphosphonate derivatives have been used in clinical practice for preventing and treating bone diseases. Recently, bone morphogenetic protein (BMP hereinafter), the TGF- $\beta$  gene superfamily comprising BMP-2 through BMP-9 and related proteins, have been reported to have bone morphogenetic activity.

Furthermore, the bone morphogenetic activity of one of those proteins called MP52 has been also reported (WO 93/16099 and WO 95/04819). A mature region of MP52 protein is considered to be a protein consisting of 120 amino acid residues having the N-terminal alanine, and its amino acid sequence is described in these publications.

A protein called GDF-5, having an analogous amino acid sequence with MP52, is also described in Nature, vol. 368, p. 639-643 (1994) and WO 94/15949.

However, these proteins can not be easily prepared in a purified form on an industrial scale.

Mammalian cell lines such as L-cells have been tried on for producing MP52 with genetic engineering technology. However, it has been found not easy to prepare MP52 in a purified form and in a high yield with the expression systems.

#### Detailed Description of the Invention

The present inventors have tried to prepare MP52 using *E. coli* on a large scale by genetic engineering technology. Briefly, the inventors have tried to prepare MP52 using *E. coli* by adding a codon encoding methionine to the DNA encoding mature region of MP52 which starts from alanine. The resultant product was not MP52 only but a mixture of MP52, a protein of 121 amino acid residues having the N-terminal methionine and a protein of 119 amino acid residues having the N-terminal alanine detached and starting from proline. It was extremely difficult to isolate pure MP52 at least with the mature region from the mixture.

5 The inventors have found that a protein in SEQ ID No.:1  
of the Sequence Listing starting from proline at the N-ter-  
minus can be selectively produced in an extremely high yield  
by constructing a plasmid wherein a codon encoding methionine  
was connected to the DNA sequence encoding amino acid sequence  
in SEQ ID No.:1 of the Sequence Listing consisting of 119  
amino acid residues with elimination of the N-terminal alanine  
of MP52, and by using the obtained plasmid-introduced *E. coli*  
for expression. Moreover, the homodimer of the protein  
described in SEQ ID No.:1 in the Sequence Listing was con-  
firmed to have a cartilage and bone morphogenetic activity,  
and thus the invention was completed.

10 An object of the invention is to provide a protein having  
amino acid sequence shown in SEQ ID No.:1 of the Sequence  
Listing. The protein consists of 119 amino acid residues, and  
corresponds to one in which the N-terminal alanine is  
eliminated from human MP52 which is regarded as a mature  
region consisting of 120 amino acid residues. The protein ac-  
cording to the invention is soluble in water. Moreover, the  
protein is low toxic itself because it is derived from human.

15 Another object of the invention is to provide a pharma-  
ceutical composition for treating cartilage and/or bone  
diseases, which comprises as an active ingredient a homodimer  
of the protein having the amino acid sequence shown in SEQ ID  
No.:1 of the Sequence Listing. More in detail, the invention  
relates to a pharmaceutical composition for preventing and  
treating osteoporosis, osteoarthritis such as gonarthrosis  
deformans and malum coxae deformans, or arthroseitis,

cartilaginous lesion such as articular meniscus lesion,  
reconstruction in the defective parts of bone and cartilage  
caused by injury and oncoectomy, defect of bone and cartilage,  
bone fracture, congenital cartilage and bone diseases such as  
5 chondrodysplasia, chondrohypoplasia, achondrogenesis,  
palatoschisis and osteodysplasia, and furthermore radicular  
and arvecular defects, since the homodimer protein according  
to the invention has a cartilage and bone morphogenetic  
activity. Furthermore, the homodimer protein can be applied  
to a treatment of bone grafting in cosmetic surgery. These  
10 treatments also include those in the area of veterinary  
surgery.

A further object of the invention is to provide a process  
for preparing a protein consisting of 119 amino acid residues  
derived from human MP52 shown in SEQ ID No.:1 of the Sequence  
15 Listing using *E. coli*.

In particular, the invention relates to the construction  
of a plasmid containing a DNA sequence encoding the amino acid  
sequence consisting of 119 amino acid residues shown in SEQ ID  
20 No.:1 of the Sequence Listing with an additional methionine at  
the N-terminus. Only the mature region of human MP52 cDNA was  
amplified by polymerase chain reaction (PCR method) by using,  
as a template DNA, a plasmid vector containing cDNA described  
in WO 93/16099. The PCR method referred to herein generally  
25 means to multiply a very small amount of fragments of DNA or  
RNA by the method described in U.S. Patent 4,683,195.

It is necessary for preparing the protein of the inven-  
tion to construct appropriate expression vectors containing

DNA encoding the protein, which are then introduced into desirable *E. coli* host strains by genetic engineering technology. The following two improved processes were applied for a large scale production of the protein;

5           1)     A process for increasing the productivity of target proteins by increasing the translation efficiency as reported by M. Nobuhara et al. (Agric. Biol. Chem., 52 (6), 1331-1338, 1988), viz. the method of increasing the AT content around the ATG initiation codon, and

10           2)     A process for increasing an average copy number of plasmids per cell, viz. the method of replacing ori region for the replication origin of pBR vector by that of pUC vector. Further, the expression vector (pKOT245) of the invention was constructed by direct ligation of the promoter region with the  
15           DNA sequence encoding amino acid sequence in SEQ ID No.:1 of the Sequence Listing with an additional methionine in its N-terminus. The *E. coli* containing said vector was deposited (Accession No. BIKOKEN-KI P-14895) at National Institute of Bioscience and Human-Technology, Agency of Industrial Science  
20           and Technology which is located at 1-3, Higashi 1-chome, Yatake-cho, Tsukuba-shi, Ibaraki-ken, 305 Japan on April 14, 1995 and transferred to a deposit (Accession No. BIKOKEN-KI BP-5499) on April 10, 1996 according to Budapest Treaty on the International Recognition of the Deposit of Microorganisms.

25           This invention relates to a process for preparing monomer proteins comprising the steps of:

          constructing a plasmid containing DNA encoding amino acid sequence in SEQ ID No.:1 of the Sequence Listing with a methi-



online at its N-terminus,

introducing the plasmid into *E. coli* for transformation,  
cultivating the *E. coli* to obtain inclusion bodies,

5     solubilizing and purifying said inclusion bodies to obtain monomer proteins, and  
to a process for preparing homodimer proteins of the protein  
in SEQ ID No.:1 of the Sequence Listing by refolding and purifying the monomer proteins obtained in the above. Briefly,  
the proteins of the invention were prepared by solubilizing  
10     the *E. coli* inclusion bodies followed by loading on SP-Sepharose FF column and Sephacryl S-200 column to obtain purified sulfonated MP52 monomers, which were subjected to refolding and isoelectric precipitation, then to RESOURCE RPC column of reverse-phase HPLC to obtain the purified dimer fractions of  
15     the proteins. The physicochemical properties of the proteins were analyzed on the basis of N-terminal amino acid sequence and amino acid composition and by electrophoresis.

20     This invention further relates to a process for culturing *E. coli* which were introduced with the expression vectors of the invention under the conditions of culture medium at 28-34°C, pH 6-8 and a dissolved oxygen concentration of 20-50%.

25     This invention further relates to a method for treating cartilage and bone diseases, which comprises administering to a human a pharmaceutical composition containing, as an active ingredient, an effective amount of the homodimer protein.

Biological activities of the homodimer protein were determined by analysis of soft X-ray radiographs, analysis of tissue-staining and analysis of time-course of ectopic carti-

lage/bone formation. Furthermore, from the results of the effect on the intramembranous ossification, the effect on the regeneration of articular cartilage and the effect on the healing of bone fracture and defects, the homodimer protein of the present invention is proved to be beneficial to the therapies of cartilage and/or bone regeneration.

The homodimer protein of the invention can be administered in systemic by intravenous, intramuscular or intra-peritoneal injection. In case of intravenous administration, an intravenous drip infusion can also be used, in addition to conventional intravenous injections.

Injectable preparations can be formulated, for example, in the form of injectable powders. In that case, the powders can be prepared by adding one or more of suitable water-soluble excipients such as mannitol, sucrose, lactose, maltose, glucose, fructose and the like, to an active ingredient, dissolving the mixture in water, dividing it into vials or ampoules followed by lyophilizing and hermetically sealing.

In the case of local administration, the homodimer protein can be coated on the surface of cartilage, bone or tooth to be treated with collagen paste, fibrin glue or other adhering materials. In the case of bone grafting, both natural bone and conventional artificial bone can be used. Artificial bone means the bone made of metal, ceramics, glass, and other natural or artificial inorganic substance. Hydroxyapatite is cited as preferable artificial substance. For example, artificial bone can be constructed by steel as dense material in the inner part and hydroxyapatite as porous material in outer part. Moreover,

it is beneficial to apply the homodimer protein to the part from which cancerous bone tissue is removed in order to accelerate the reconstruction of bone. It can also be applied to the cartilage grafting.

5           The dose may be varied depending upon various factors influencing the activity of the protein such as weight of bone and cartilage to be reconstructed, injured site of bone and cartilage and the symptoms, age and sex of patients, severity of infection, administration intervals and other clinical factors. The dose can also be varied depending upon types of carriers to be used for restructuring with the dimer protein. In general, the dose is in the range of about  $10-10^6$  ng of the homodimer protein per wet weight of desired bone and cartilage when administered as a composition containing a carrier. In the case of local and systemic administration by injection, it is preferable to administer  $0.1-10^4$   $\mu$ g in a frequency of from once a week to once a day.

10           A synergetic effect can be expected by administering the homodimer protein simultaneously with known growth factors, for example, insulin-like growth factor-I for regeneration of bone and cartilage.

15           There has never been reported of a process for preparing the protein of the invention on an industrial scale and in a purified form as described above, and the homodimer protein is useful as a medical composition for treating cartilage and bone diseases since it has a cartilage and bone morphogenetic activity. Further, the process of preparing the protein of the present invention can be applicable for the preparation of other

proteins of the above-described TGF- $\beta$  superfamily members, all of which were only successful so far to prepare by using mammalian cell lines.

This invention is further illustrated by the following examples. However, it should not be construed that the invention is limited to these specific examples.

### Example

#### Example 1 Construction of expression vector

##### (1) Isolation of a mature region of MP52

A mature region of human MP52 cDNA was PCR-amplified using the plasmid vector (pSK52s) containing cDNA described in WO 93/16099 as a template DNA.

In accordance with the process for increasing a productivity of the target proteins reported by M. Nobuhara, et al. {Agric. Biol. Chem., 52 (6), 1331-1338, 1988}, a part of DNA of the mature region of MP52 gene was substituted to increase the AT content around the ATG initiation codon.

The mutagenesis was introduced by PCR method using the designed upstream PCR primer encompassing the mutation of SEQ ID No.:2 of the Sequence Listing. For the DNA sequence of the PCR primers were used the DNA in the SEQ ID No.:2 as an upstream primer, and the DNA in SEQ ID No.:3 of the Sequence Listing as a downstream primer.

The PCR was performed by adding the template DNA (10 ng), 50 pmols each of the PCR primers in an order direction and in a reverse direction, dNTP (0.2 mmol) and MgCl<sub>2</sub> (1.5 mmol) in the same test tube, together with Taq DNA polymerase (5 U).

Thirty cycles of PCR were performed; the conditions of each cycle were 94°C for a minute for denaturation, 55°C for a minute for primer annealing, and 72°C for 2 minutes for primer extension.

5           The products obtained from the PCR was isolated by electrophoresis in 1.5% low melting point agarose (purchased from FMC), and the fragments of about 360 bp were isolated (Fragment 1).

(2)   Construction of *E. coli* expression vector for the  
10           protein of the invention

15           In order to increase a copy number of the plasmid per bacteria, the *ori* region for replication origin was changed from that of pBR to pUC vector. The *E. coli* expression vector pKK223-3 available in the market (purchased from Pharmacia Biotech) was used to isolate *tac* promoter region by digestion with restriction endonucleases *SspI* and *EcoRI*, and also to isolate *rrnBt<sub>1</sub>t<sub>2</sub>* terminator region by using *SalI* and *SspI*. A DNA fragment of *tac* promoter region which had been treated with Mung Bean Nuclease (Takara Shuzo Co., Ltd.) was ligated by T4  
20           DNA ligase with Fragment 1 which was obtained above. The resultant DNA fragment was digested by *SalI* and re-ligated with the *rrnBt<sub>1</sub>t<sub>2</sub>* region. The DNA fragment was ligated into the *SmaI* site of pUC18 vector to construct the expression vector {pKOT245 (Accession No. BIKOKEN-KI P-14895)} (Fig. 1) for the production  
25           of the protein. The length of pKOT245 DNA is 3.7 kb. The nucleotide sequence of the expression vector constructed for the protein was analyzed by Pharmacia ALF DNA sequencer.

### (3) Transformation

Transformation was performed according to the rubidium chloride transformation method by Kushner *et al.* (Genetic Engineering, p. 17, Elsevier, 1978). Briefly, pKOT245 was used to transform the host strain *E. coli* W3110M according to the method described above to produce *E. coli* transformants for the production of the protein.

### Example 2 Cultivation

#### (1) Cultivation

The *E. coli* expressing the protein of the invention was precultured in the modified SOC medium (Bacto tryptone 20 g/l, Bacto yeast extract 5 g/l, NaCl 0.5 g/l,  $MgCl_2 \cdot 6H_2O$  2.03 g/l, Glucose 3.6 g/l). 100 ml of the bacteria suspension was used to inoculate 5 l of the production medium (Bacto tryptone 5 g/l, Citric acid 4.3 g/l,  $K_2HPO_4$  4.675 g/l,  $KH_2PO_4$  1.275 g/l, NaCl 0.865 g/l,  $FeSO_4 \cdot 7H_2O$  100 mg/l,  $CuSO_4 \cdot 5H_2O$  1 mg/l,  $MnSO_4 \cdot nH_2O$  0.5 mg/l,  $CaCl_2 \cdot 2H_2O$  2 mg/l,  $Na_2B_4O_7 \cdot 10H_2O$  0.225 mg/l,  $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$  0.1 mg/l,  $ZnSO_4 \cdot 7H_2O$  2.25 mg/l,  $CoCl_2 \cdot 6H_2O$  6 mg/l,  $MgSO_4 \cdot 7H_2O$  2.2 g/l, Thiamine HCl 5.0 mg/l, Glucose 3 g/l), which was cultured in a 10-liter fermentor with aeration-agitation, and then upon reaching the early stage of logarithmic growth phase ( $OD_{550}=5.0$ ), isopropyl-3-D-thio-galactopyranoside at a final concentration of 1 mM was added and the cultivation was continued until reaching  $OD_{550}=150$ . During the cultivation, temperature was kept at 32°C, and pH value of 7.15 by adding ammonia. In order to prevent lowering of a dissolved oxygen concentration, an agitation was sped up to keep the dissolved

oxygen concentration at 50% of air saturation. The cultivation was proceeded by adding 50% glucose solution at a level of 0.2% to obtain a high cell density, with an indication of abrupt increase of the dissolved oxygen concentration.

5

(2) Preparation of *E. coli* inclusion bodies

The culture broth obtained by the method described above was centrifuged to harvest the cells, which were then suspended in 25 mM Tris-HCl buffer containing 10 mM ethylene diamine tetraacetic acid (pH 7.3). The cells were disrupted by passing through a homogenizer (made by APV Gaulin Inc.) and centrifuged again to harvest the precipitate containing the inclusion bodies.

10

Example 3 Purification

(1) Solubilization of *E. coli* inclusion bodies

After washing with 1% Triton X-100 three times, the *E. coli* inclusion bodies were centrifuged at 3,000 x g for 30 minutes at 4°C, and then the resultant precipitate was solubilized by sonication in 20 mM Tris-HCl buffer, pH 8.3, 8 M urea, 10 mM DTT, and 1 mM EDTA.

15

20

(2) Preparation of monomers

The solubilized solution was centrifuged at 20,000 x g for 30 minutes at 4°C and the resultant supernatant was collected. The obtained supernatant was subjected to SP-Sepharose FF (Pharmacia AB) equilibrated with 20 mM Tris-HCl buffer pH 8.3, 6 M urea, and 1 mM EDTA, and then, after washing with the same

25

solution, it was eluted with the same solution containing 0.5 M NaCl. The protein in the eluate were sulfonated by adding  $\text{Na}_2\text{SO}_3$  and  $\text{Na}_2\text{S}_4\text{O}_6$  to reach the final concentration respectively at 111 mM and 13 mM and by incubating at 4°C for 15 hours. The sulfonated solution was gel-filtrated on Sephacryl S-200 HR (Pharmacia AB) equilibrated with 20 mM Tris-HCl buffer, pH 8.3, 6 M urea, 0.2 M NaCl, and 1 mM EDTA to obtain purified sulfonated monomers of the protein of the invention.

### (3) Refolding

The solution of the sulfonated monomers was added into a 9 times volume of 50 mM Na-Glycine buffer pH 9.8, 0.2 M NaCl, 16 mM CHAPS, 5 mM EDTA, 2 mM GSH (reduction type glutathione), and 1 mM GSSG (oxydation type glutathione) with stirring, and then incubated for 24 hours at 4°C to oxidize and refold the protein of the invention.

### (4) Preparation of homodimers

The refolding solution was diluted with the same volume of purified water and then by adding 6 N NaCl adjusted pH value to approximately 7.4 and placed to isoelectric precipitation. The precipitates collected by centrifugation at 3,000 x g for 20 minutes were solubilized in a solution with 30% acetonitrile containing 0.1% TFA. The solution was diluted with the same volume of purified water and loaded on RESOURCE RPC column (Pharmacia AB) of a reverse-phase HPLC preequilibrated with 25% acetonitrile containing 0.05% TFA, and then eluted with a linear gradient of 25-45% acetonitrile containing 0.05% TFA. The



eluate was monitored at 280 nm absorbance. The purified homodimer protein fractions were collected and lyophilized by SpeedVac Concentrator (Servant Co.)

(5) Determination of physicochemical properties of the purified protein of the invention

a) Analysis of N-terminal amino acid sequence

Analysis of the N-terminal amino acid sequence for the purified proteins was performed using an amino acid sequencer Model 476A (Applied Biosystems Inc.) to confirm the amino acid sequence from the N-terminal to the 30th amino acid as shown in SEQ ID No.:1 of the Sequence Listing.

b) Analysis of amino acid composition

The analysis of amino acid composition of the purified proteins obtained above was performed by an amino acid sequencer (PICO TAG Systems, Waters). The result was shown in Table 1. The number described in Table 1 indicates the number of amino acid residue per a monomer protein.

Table 1

Amino acid	Practical number	Expected number
Asx	11.5	12
Glx	10.9	11
Ser	8.4	9
Gly	4.3	4
His	4.0	4
Arg	7.7	7
Thr	5.4	6
Ala	7.3	7
Pro	10.2	10
Tyr	2.9	3
Val	5.7	7
Met	5.1	4
1/2Cys	2.6	7
Ile	4.9	6
Leu	10.0	10
Phe	4.0	4

Lys	5.9	6
Typ	-	2

length of the sequence	119
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5 -: undetectable

#### c) Analysis by electrophoresis

Molecular weight of the purified proteins obtained above was confirmed to be about 28 KDa on SDS-PAGE under non-reducing condition.

10 From the results shown in the above a), b) and c), it is found that the protein of the invention comprises 119 amino acid residues starting from the N-terminal Pro singly.

#### 5 Example 4 Determination of biological activities

##### (1) Activity in ectopic bone formation in mice.

About 500 µg of the homodimer protein obtained in Example 3 was dissolved and diluted in 50 µl of 10 mM hydrochloric acid, and 1 µg/10 µl, 10 µg/10 µl, and 100 µg/10 µl concentrations of the solution were prepared. Ten µl of each solution was mixed with 150 µl porcine tendon type-I collagen solution (Koken, 0.5%, pH 3, I-AC), neutralized, lyophilized, and the resultant mixture was implanted into pockets created in the thigh muscles of 8-week-old male ICR mice. At day 21 from implantation, the animals were sacrificed and the thighs were excised. After peeling skins off, the incidence of calcified tissues was evaluated by soft X-ray radiography. As shown in Table 2, the implantation of 1 µg/site or more of the dimer protein induced calcified tissue in part of the group of the mice, and 10 and more doses

induced calcified tissue in all mice used.

Table 2

Dose of the homodimer protein	Incidence of calcified tissue
Control (Type-I collagen alone)	0/4
1 µg/site	3/4
10 µg/site	4/4
100 µg/site	4/4

Figure 2 shows typical examples of soft X-ray radiographs of calcified tissue induced by different doses of MP52 protein. Figures 2A, 2B and 2C show examples of soft X-ray radiographs of 1 µg the homodimer protein/site-, 10 µg/site- and 100 µg/site-implanted mice thighs, respectively. These radiographs indicate that the homodimer protein induced calcified tissue in the mouse thigh and increased it in a dose-dependent manner. In order to verify if the formed calcified tissues were cartilage or bone, the sections of the fixed mouse thighs into which 10 µg/site the homodimer protein was implanted were stained with von Kossa, Alcian blue or Hematoxylin-eosin.

Figure 3 shows light-microscopic photographs of the sections stained with the respective staining methods. In Figure 3A (von Kossa staining), areas indicated by ct and cc show calcified tissue and calcified chondrocytes, respectively. In Figure 3B (Alcian blue staining), an area indicated by rc shows remaining cartilage tissue. In Figure 3C (Hematoxylin-eosin staining), elements indicated by ad, bm,

lb, ob and wb are an adipocyte, bone marrow cells, lamellae bone, osteoblasts, and woven bone, respectively. Thus, it is evident that the implantation of the homodimer protein with Type-I collagen into mouse thighs induces calcified chondrocytes, osteoblasts, and bone marrow cells at the sites.

Thus, the homodimer protein was demonstrated to possess activity in ectopic cartilage and bone formation.

(2) Analysis of time-course of ectopic bone formation in mice

The dimer protein (3  $\mu$ g) obtained in Example 3 was mixed with Type-I collagen solution and neutralized as described in Example 4 (1), and the lyophilized materials were implanted into the male ICR mouse thighs. At days 3, 7, 10, 14, 21, and 28 from implantation, the thighs were excised and fixed in 10 % formalin, and then, sections were stained with Hematoxylin-eosin or von Kossa. Figure 4 shows the light-microscopic photographs of the sections stained.

At day 3 (Figure 4A, Hematoxylin-eosin staining), undifferentiated mesenchymal cells (mc) including morphologically fibrous connective cells appeared in the space between collagen fibers (co) implanted and muscle cells (m). At between days 7 and 10 (Figures 4B and 4C, respectively, Hematoxylin-eosin staining), the space was filled with undifferentiated mesenchymal cells (mc) and these cells were hypertrophied and differentiated into precartilagenous tissue. At day 14 (Figure 4D, Hematoxylin-eosin staining and Figure 4E, von Kossa staining), calcified cartilage tissue (cc) and bone tissue (b) were observed. At day 21 (Figure 4D,

Hematoxylin-eosin staining and Figure 4E, von Kossa staining), calcified cartilage tissue was not observed at all, and the tissue observed at day 14 appeared to be replaced into bone (b) with bone marrow (bm). At day 28 (Figure 4H, Hematoxylin-eosin staining), there were a large mount of bone marrow cells and formed bone appeared to be under a resorptive process.

Thus, it is evident that the homodimer protein induces endochondral ossification through cartilage formation at ectopic sites, as reported by using other BMPs.

### (3) Effect on the intramembranous ossification

The homodimer protein obtained in Example 3 was dissolved in phosphate-buffered saline (pH 3.4 ) containing 0.01% human serum albumin, and 0.01  $\mu\text{g}/20\ \mu\text{l}$ -, 0.1  $\mu\text{g}/20\ \mu\text{l}$ -, and 1  $\mu\text{g}/20\ \mu\text{l}$ -concentrations of solutions were prepared. Twenty  $\mu\text{l}$ -portion of each solution was injected 12 times once a day onto the periosteum of neonatal rat parietal bone by using a microsyringe from day 1 after birth. The same volume of the vehicle was injected onto the counter-side of parietal bone of each rat. The same volume of the vehicle was also injected onto both sides of parietal bones of control rats. At day 1 from the final injection, the rats were sacrificed and the both sides of parietal bones were excised and fixed, and then, the decalcified sections stained with Hematoxylin-eosin were prepared to measure the thickness of the parietal bones at the injected sites on microscopic photographs. The ratio of the homodimer protein-injected site/vehicle-injected site in the parietal bone thickness of each rat was calculated. As shown

in Table 3, the homodimer protein increased parietal bone thickness in a dose-dependent manner. A typical example of microscopic photographs of the section at a homodimer protein 0.1 µg/site-injected site is shown in Figure 5B in comparison with that of the counter-side of vehicle-injected site (Figure 5A). The injection of the homodimer protein induced the activation and proliferation of periosteal cells (p), and activated osteoblasts (ob) were observed in and on the parietal bone (b). These results indicate that the homodimer protein stimulated intramembranous ossification when locally injected, and that the homodimer protein is beneficial to the therapies of osteoporosis, bone fracture, and alveolar ridge and periodontal defects.

Table 3

Dose of homodimer protein protein (µg/site/day)	Parietal bone thickness (µm)		Ratio (B/A)
	vehicle-injected site (A)	VP52-injected site (B)	
0 (vehicle)	128 ± 7	141 ± 20	1.10 ± 0.16
0.01	134 ± 9	167 ± 30	1.27 ± 0.33
0.1	119 ± 19	190 ± 29	1.60 ± 0.10*
1	132 ± 9	225 ± 25	1.70 ± 0.14**

Values represent means ± SD (n=4), \*p<0.05, \*\*p<0.01 vs. ratio of the group in which vehicle was injected into both the sites (Williams' test).

#### (4) Effect on the regeneration of articular cartilage

Six 12-week-old male New Zealand White rabbits were used for this study. Right knee skin and articular capsule were cut and a 5 x 5 mm full thickness osteochondral defect was created in the patellar groove using a dental burr so as not to damage surrounding tendons. The defects were filled with

either lyophilized Type-I collagen sponge or with lyophilized Type-I collagen sponge containing 10 µg homodimer protein, prepared as described in Example 4 (1), and then, the cut articular capsule and skin were sutured. Three weeks post-operatively, the rabbits were sacrificed and the femoral heads were excised and fixed in 10% formalin, and then, decalcified sections were stained with Alcian blue. Typical examples of microscopic photographs of the sections were shown in Figure 6. The dimer protein treated defects (Figures 6C and 6D) demonstrated the regeneration of chondrocytes (ch) with extracellular matrices which were stained intensively with Alcian blue, as compared to the Type-I collagen sponge implanted control defects (Figures 6A and 6B) which were filled with fibrous tissue (f). The cartilage tissue induced by the dimer protein showed zonal structure including resting chondrocytes, growing chondrocytes and hypertrophied chondrocytes, like that of normal articular cartilage. The chondroinduction by the MP52 protein were observed in the defects of all rabbits used (n=3). These results indicate that the dimer protein is effective to the repair of damaged cartilage tissue in patients such as osteoarthritis.

#### (5) Effect on the healing of bone fracture and defects

Thirty male Sprague-Dawley rats (about 15-week-old) were used for this study. Using a lateral approach to the femur, all muscle and periosteal tissue were stripped from the diaphysis. A 5 mm-segmental bone defect was created in the middle region of the right femur shaft with use of dental

burr, and then, a special-made polyethylene plate was fixed with stainless screws along the cortex of the femur. Type-I collagen sponges containing 0, 1, 10, and 100  $\mu$ g of the homodimer protein were prepared as described in Example 4 (1), and implanted into the segmental bone defects and then, the wound was sutured. Just after operation and 12 weeks post-operatively, the defects were evaluated by soft X-ray radiography. As shown in Figure 7, 10 and 100  $\mu$ g/site of the homodimer protein stimulated callus (cs) formation in the defects and formed bony unions, but the effect at 1  $\mu$ g/site was not clear as compared to the control collagen implanted defect in which only marginal endosteal bone formation was observed. Twelve weeks post-operatively, rats were sacrificed, and the femur with a defect was excised and bone mineral content (accumulated one of mid-three scanings in the defect) was measured by dual energy X-ray absorptiometry (Aloka, Model DCS-600) in a mode with 1 mm scanning width after removing the polyethylene plate. Both ends of the femur with the resin were fixed, then, maximum torsional strength to break the union of specimens were measured by bone strainer system (Malto, model MZ-500D) in a routing speed with 180°/min (Table 4). It shows that the homodimer protein increases both bone mineral content and bone strength at the rat femur defect in which the protein is implanted, and indicates the efficacy of the present protein for fracture healing and bone reconstruction of the defect.



Table 4

	Dose of homodimer protein ( $\mu\text{g}/\text{site}$ )	Bone Mineral Content in rat femur defect (mg)	Maximum Torsional Strength (Kgf $\cdot\text{cm}$ )	Number
5	collagen alone	120.2 $\pm$ 24.5	2.92 $\pm$ 0.09	6
	1	176.9 $\pm$ 36.4	6.24 $\pm$ 1.00	8
	10	277.4 $\pm$ 63.9	9.35 $\pm$ 3.14	8
	100	374.8 $\pm$ 67.1*	40.34 $\pm$ 7.64*	8

Values represent means  $\pm$  SE, \* $p$ <0.05 vs. collagen alone group  
(Student's t-test).

From the results in Example 4, the homodimer protein of the invention was found to have a cartilage and bone morphogenetic activity.

The protein composed of a homodimer of the protein having an amino acid sequence in SEQ ID No.:1 of the Sequence Listing has a cartilage and bone morphogenetic activity and is useful as a pharmaceutical composition for treating cartilage and bone diseases. Furthermore, the protein of the invention can be prepared on an industrial scale and in a pure form by a gene engineering process culturing *E. coli* transformed with a higher copy number expression vector for said protein.

"Sequence Listing"

SEQ ID No.:1

Sequence length:119

5 Sequence type:amino acid

Topology:linear

Molecular Type:peptide

Type of Fragment:N-terminal fragment

Original Source

10 Organism:homo sapiens

Name of tissue:fetus

Features:

Other information: From 383 to 501 amino acid sequence  
of MP52 amino acid sequence

15 Sequence Description:SEQ ID No.:1:

CCA CTG GCC ACT CGC CAG GGC AAG CGA CCC AGC AAG AAC CTT AAG GCT 48

Pro Leu Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala

20 5 10 15

CGC TGC AGT CGG AAG GCA CTG CAT GTC AAC TTC AAG GAC ATG GGC TGG 96

Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp

25 20 25 30

GAC GAC TGG ATC ATC GCA CCC CTT GAG TAC GAG GCT TTC CAC TGC GAG 144

30 Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu

35 40 45

35 GGG CTG TGC GAG TTC CCA TTG CGC TCC CAC CTG GAG CCC ACG AAT CAT 192

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His

50 55 60

40 GCA GTC ATC CAG ACC CTG ATG AAC TCC ATG GAC CCC GAG TCC ACA CCA 240

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro

45 65 70 75 80

CCC ACC TGC TGT GTG CCC ACG CGA CTG AGT CCC ATC AGC ATC CTC TTC 288

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe

50 85 90 95

ATT GAC TCT GCC AAC AAC GTG GTG TAT AAG CAG TAT GAG GAC ATG GTC 336

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val

5 100 105 110

GTG GAG TCG TGT GGC TGC AGG 357

Val Glu Ser Cys Gly Cys Arg

10 115

SEQ ID No.:2

Sequence length:27

15 Sequence type:nucleic acid

Strandness:single

Topology:linear

Molecular Type:other nucleic acid

Original Source:none

20 Organism:none

Strain:none

Features:PCR upstream primer of MP52 mature type

Sequence Description:SEQ ID No.:2:

ATAATGCCAC TAGCAACTCG TCAGGGC 27

25

SEQ ID No.:3

Sequence length:26

Sequence type:nucleic acid

Strandness:single

30 Topology:linear

Molecular Type:other nucleic acid

Original Source:none

Organism:none

Name of tissue:none

35 Features:

Other information:PCR downstream primer of MP52 mature type

Sequence Description:SEQ ID No.:3:

CGTCGACTAC CTGCAGCCAC ACGACT 26

#### Brief Explanation of Drawings

Fig. 1 shows a plasmid map of the expression vector (pKOT245) for the protein of the invention obtained in Example 1 (2).

5 Fig. 2 shows a soft X-ray radiograph of the calcified tissue induced in mouse thigh in Example 4 (1).

Fig. 3 shows a light-microscopic photograph of the calcified tissue stained in mouse thigh in Example 4 (1).

10 Fig. 4 shows a light-microscopic photograph of the time-coursed calcified tissue stained in mouse thigh in Example 4 (2).

Fig. 5 shows a light-microscopic photograph of rat parietal bone stained in Example 4 (3).

15 Fig. 6 shows a light-microscopic photograph of articular cartilage defects stained in the rabbit femoral head in Example 4 (4).

Fig. 7 shows a soft X-ray radiograph of the bone formation in the bone defects of the rat femurs in Example 4 (5).

What is claimed is:

1. A protein having amino acid sequence in SEQ ID No.:1 of the Sequence Listing.

2. A homodimer protein according to claim 1.

3. A pharmaceutical composition for treating cartilage and bone diseases comprising a therapeutically effective amount of the protein according to claim 2 and a pharmaceutical carrier.

4. The pharmaceutical composition according to claim 3 wherein cartilage and bone diseases are osteoporosis.

5. The pharmaceutical composition according to claim 3 wherein cartilage and bone diseases are osteoarthritis or arthroseitis.

6. The pharmaceutical composition according to claim 3 wherein cartilage and bone diseases are bone fracture and bone defect.

7. The pharmaceutical composition according to claim 3 wherein cartilage and bone diseases are radicular and arvecular defects.

8. A process for preparing the protein of claim 1 which comprises culturing *E. coli* transformed with a plasmid containing DNA sequence which is capable of expressing said protein.

9. The process according to claim 8 wherein the plasmid contains a DNA coding amino acid sequence in SEQ ID No.:1 of the Sequence Listing with a methionine at the N-terminus.

10. A process for preparing the dimer protein according to claim 2 which comprises the steps of;

constructing a plasmid containing DNA coding amino acid sequence in SEQ ID No.:1 of the Sequence Listing with a methionine at the N-terminus,

introducing the plasmid into *E. coli* for transformation, solubilizing inclusion bodies obtained by culturing said *E. coli*,

purifying the monomer protein from the solubilized solution,

refolding the monomer protein into a dimer protein and purifying the same.

11. A method for treating cartilage and bone diseases, which comprises administering to a human a pharmaceutical composition containing, as an active ingredient, an effective amount of the homodimer protein as claimed in claim 2.

12. The method for treating cartilage and bone diseases as claimed in claim 11, wherein said disease is osteoporosis.

13. The method for treating cartilage and bone diseases as claimed in claim 11, wherein said disease is osteoarthritis or arthroseitis.

14. The method for treating cartilage and bone diseases as claimed in claim 11, wherein said disease is bone fracture and bone defect.

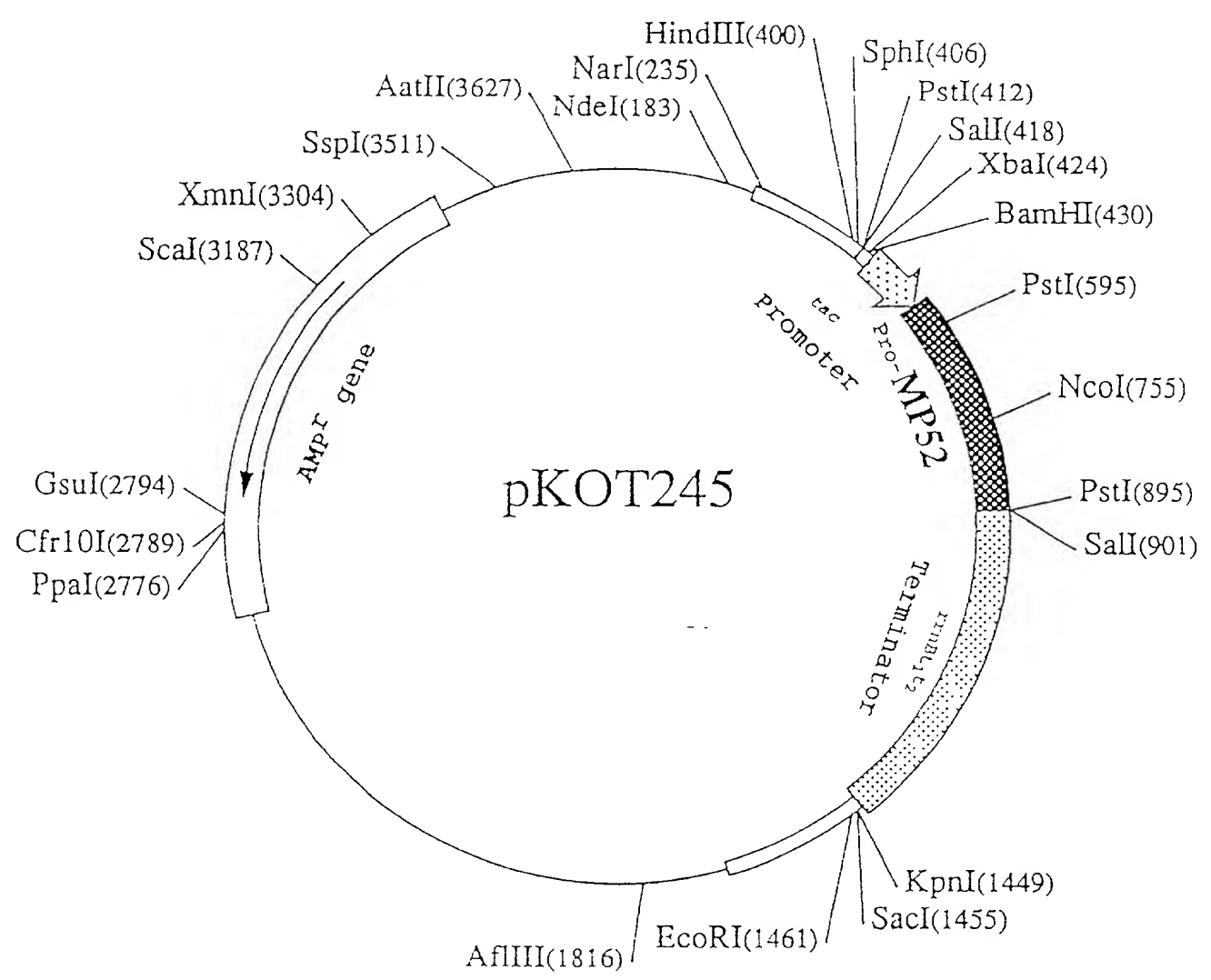
15. The method for treating cartilage and bone diseases as claimed in claim 11, wherein said disease is radicular and arvecular defects.

# Abstract of the Disclosure

5 A protein having amino acid sequence in SEQ ID No.:1 of  
the Sequence Listing derived from human MP52, and a dimer  
protein thereof. A homodimer protein described above can be  
obtained by constructing a plasmid containing DNA coding amino  
acid sequence in SEQ ID No.:1 of the Sequence Listing with a  
methionine at the N-terminus, introducing the plasmid into *E.*  
10 *coli* for transformation, solubilizing inclusion bodies  
obtained by culturing the transformant, purifying the monomer  
protein from the solubilized solution, refolding the monomer  
protein into a dimer protein and purifying the same.

15 The homodimer protein described above is useful as a  
pharmaceutical composition for treating cartilage and bone  
diseases.

Fig. 1

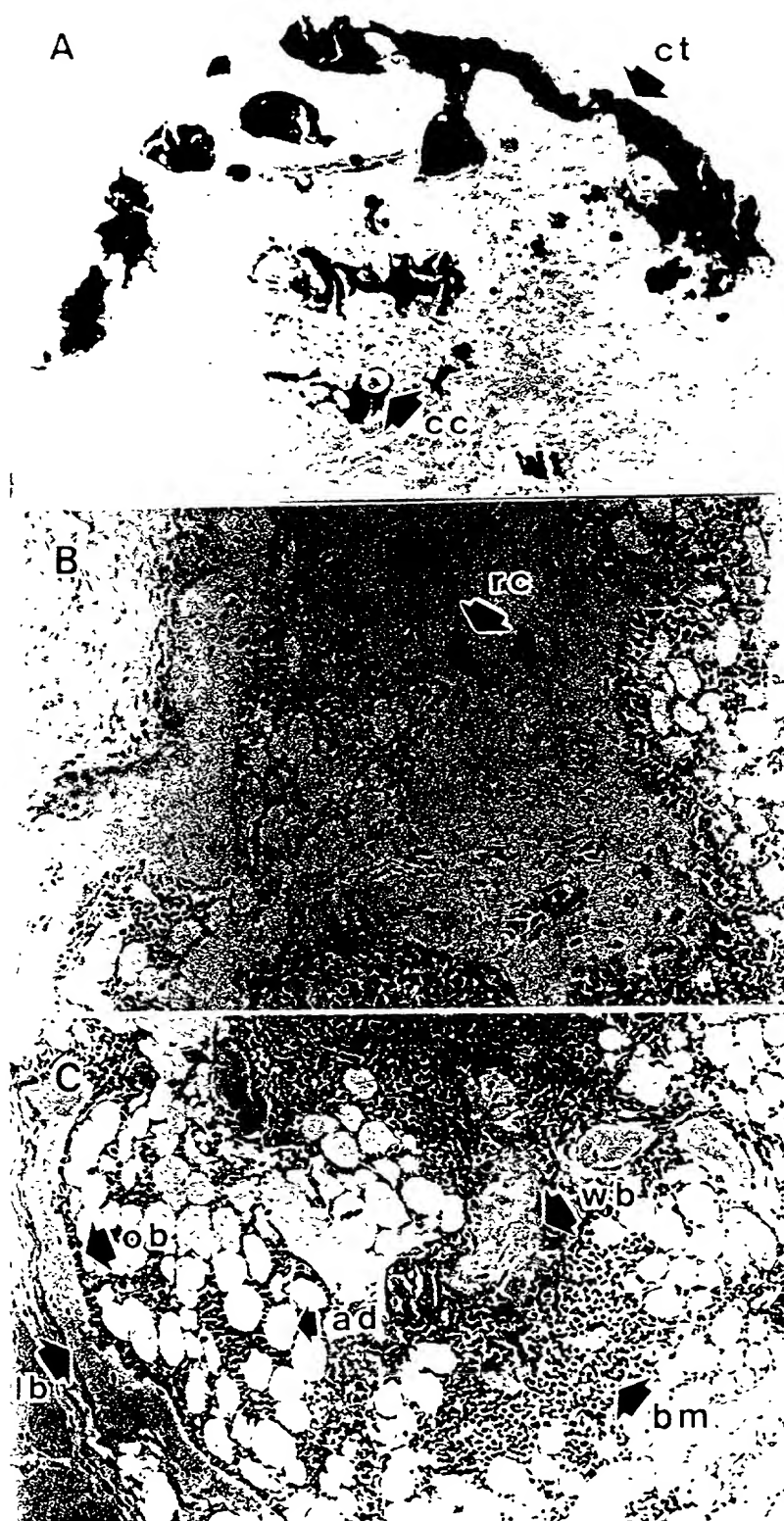






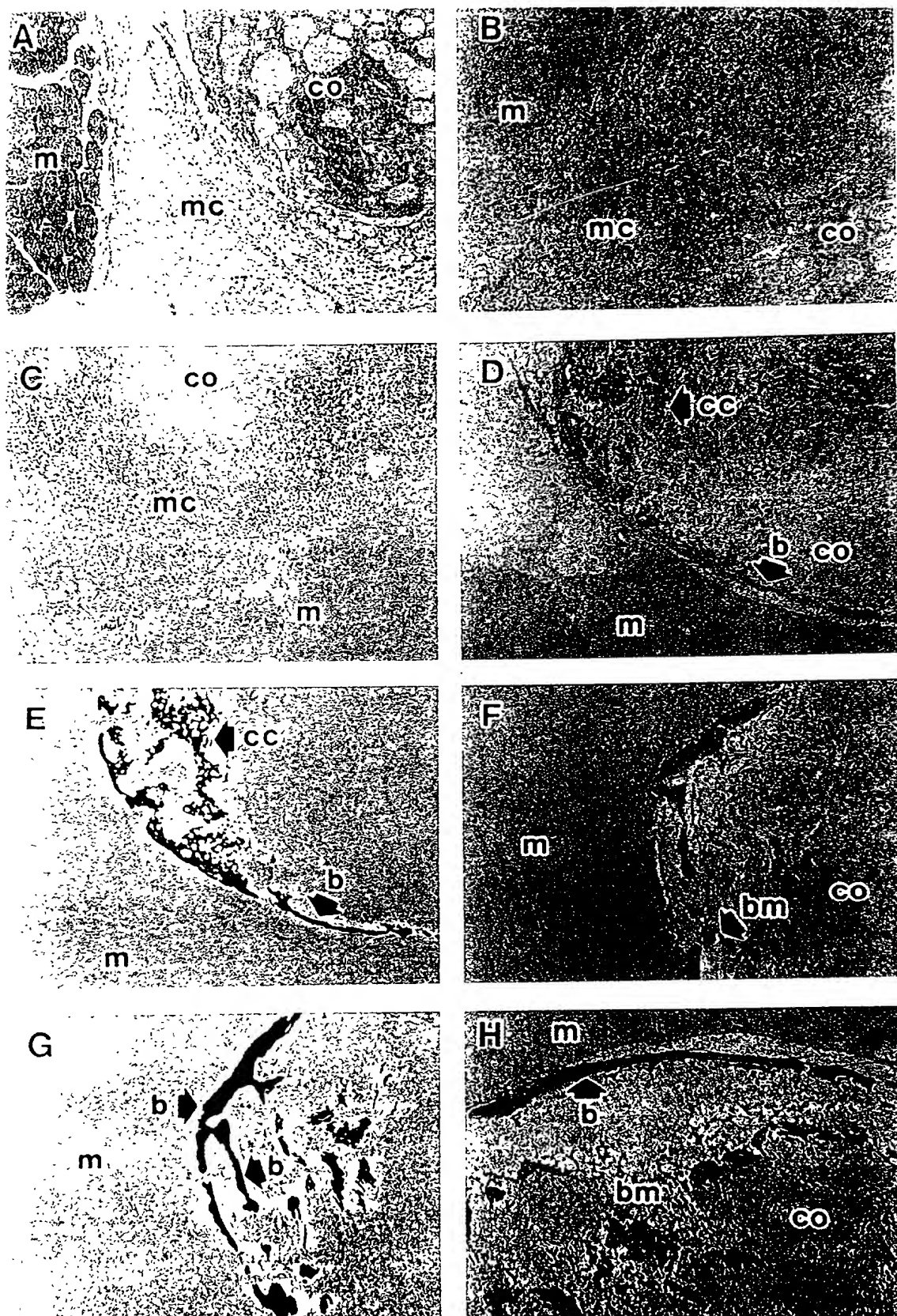
— 第 2 — 图 —

Fig. 2

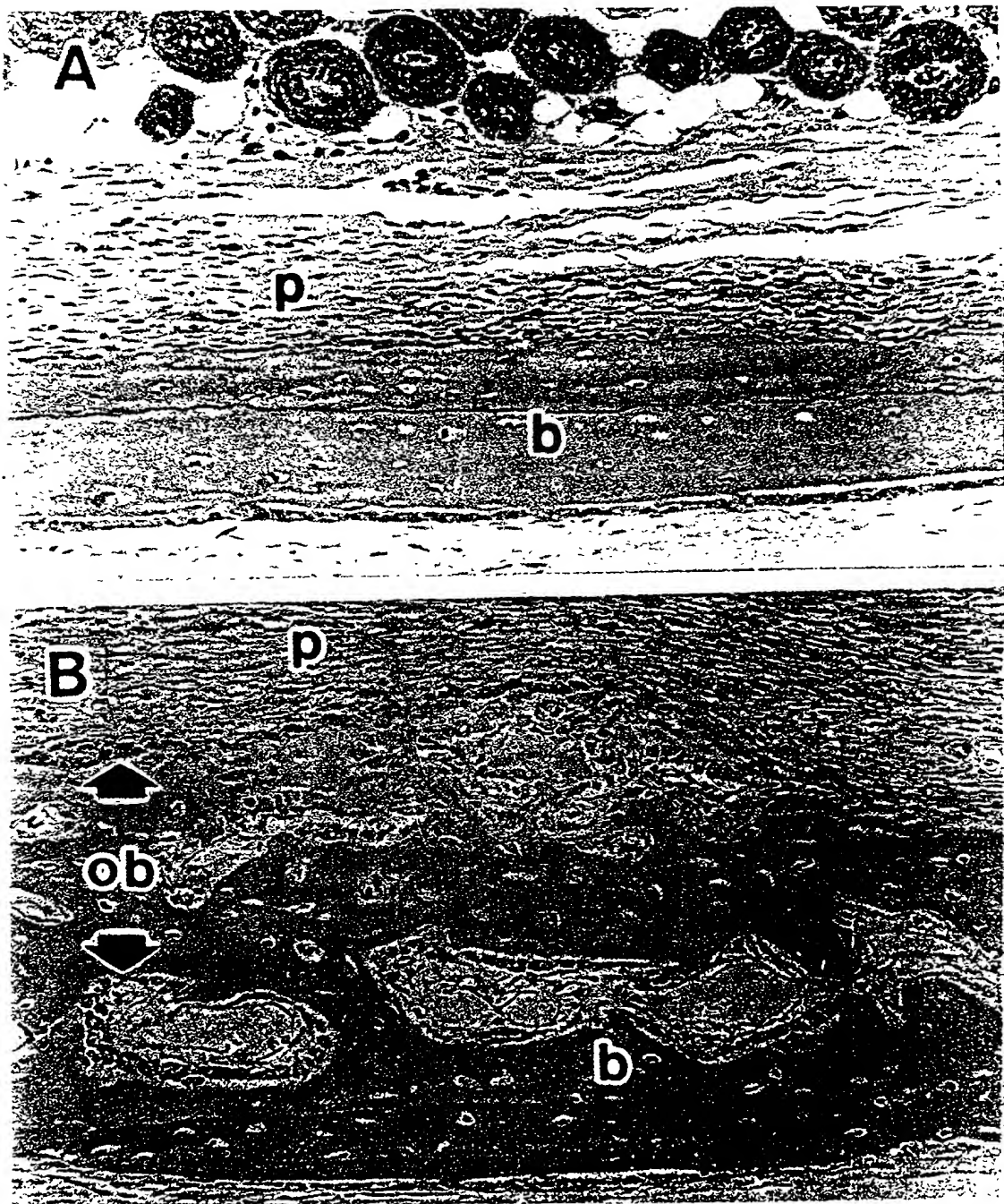


— 第 3 — 图 —

Fig. 3

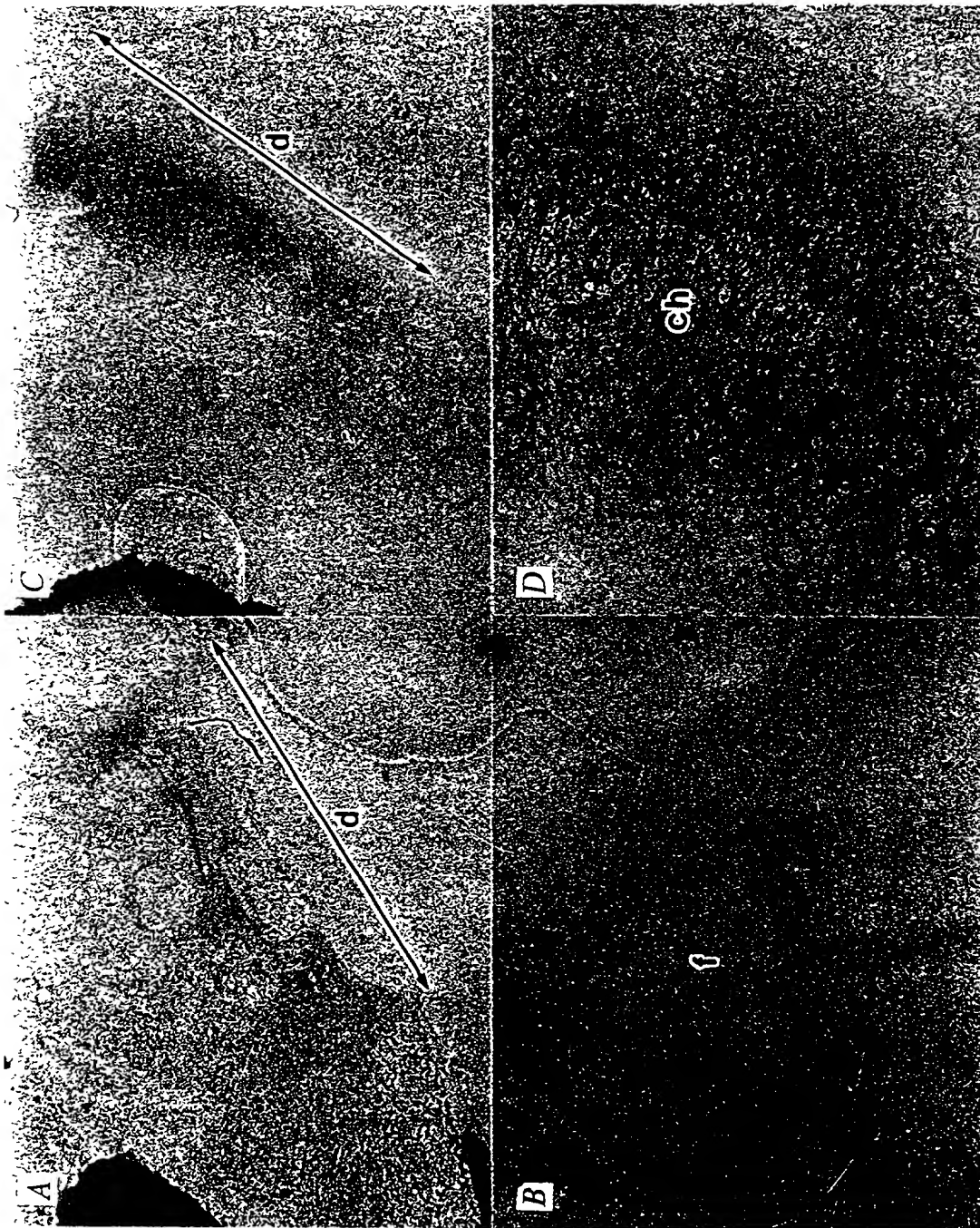


—第 4 —  
Fig. 4



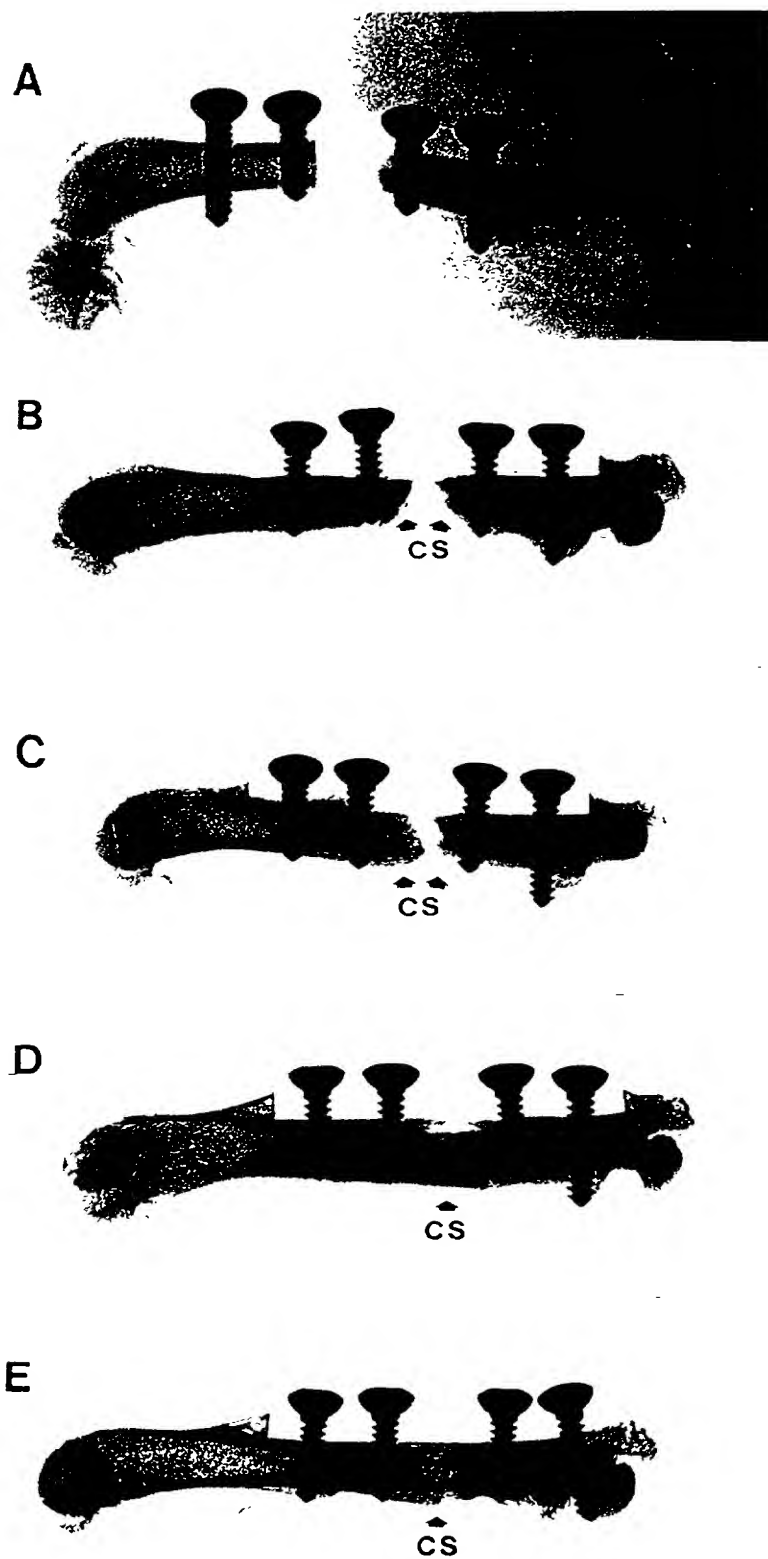
—第 5 — 图—

Fig. 5



第 6 图

Fig. 6



## COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

146.1275

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NOVEL PROTEIN AND PROCESS FOR PREPARING THE SAME

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. \_\_\_\_\_

on \_\_\_\_\_

and was amended

on \_\_\_\_\_ (if applicable).

☒ was filed as PCT international application

Number PCT/JP96/01062

on April 19, 1996

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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COUNTRY (If PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Japan	7/93664	April 19, 1995	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
Japan	7/322403	November 17, 1995	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
Japan	PCT/JP96/01062	April 19, 1996	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO



## Combined Declaration For Patent Application and Power of Attorney (Continued)

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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

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PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number) Bierman, Muserlian and Lucas, Reg. No. 18,818; Jordan B. Bierman, Reg. No. 18,629; Charles A. Muserlian, Reg. No. 19,683; and Donald C. Lucas, Reg. No. 31,275; all of 600 Third Avenue, New York, N.Y. 10016

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SIGNATURE OF INVENTOR 201 <u>Fusao Makishima</u>	SIGNATURE OF INVENTOR 202 <u>Hiroyuki Takamatsu</u>	SIGNATURE OF INVENTOR 203 <u>Hideo Miki</u>
DATE <u>November 4, 1997</u>	DATE <u>November 4, 1997</u>	DATE <u>November 4, 1997</u>



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PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED (if any)			

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	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
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SIGNATURE OF INVENTOR 201

Shinji Kawai

DATE November 7, 1997.

SIGNATURE OF INVENTOR 202

Michio Kunioka

DATE November 4 1997

SIGNATURE OF INVENTOR 203

To make it that small

DATE November 14 1994

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
Mieko Katsura	Koichi Enomoto	Yusuke Satoh
DATE	DATE	DATE
Nov. 4, 1997	November 4, 1997	November 4, 1997